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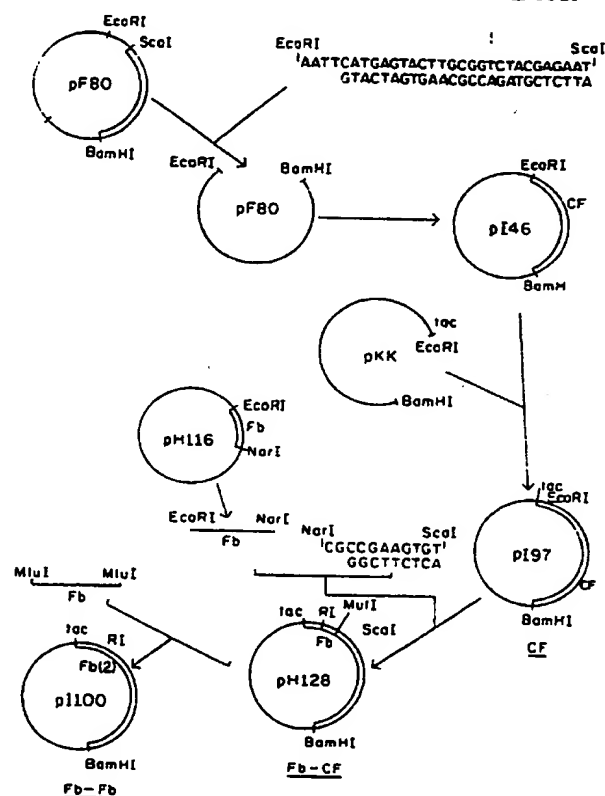
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(54) Title: PROTEIN ANALOGUES OF TISSUE PLASMINOGEN ACTIVATOR

(57) Abstract

Protein analogues of tissue plasminogen activator (tPA). The analogues exhibit the proteolytic function of natural tPA and optionally fibrin binding activity, but are molecules, generally of lower molecular weight than natural tPA, designed for efficient expression in prokaryotic host cell systems. The analogues can comprises a catalytic fragment of tPA or a catalytic fragment of tPA linked to a polypeptide which stabilizes the catalytic fragment, provides for efficient expression of the fragment or confers a fibrin binding capability. Fibrin binding polypeptides can be polypeptide fragments derived from tPA which embody the fibrin binding domain(s) of natural tPA or they can be an exogenous (non-tPA) polypeptides of eukaryotic or prokaryotic origin which exhibit fibrin binding affinity such as the antigen binding fragment of an antifibrin immunoglobulin or the B domain of protein A. Genetic constructs for expression of the analogues are also provided.

CONSTRUCTION OF EXPRESSION VECTORS IN E. COLI



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PROTEIN ANALOGUES OF TISSUE
PLASMINOGEN ACTIVATOR

Technical Field

05 This invention is in the fields of genetic and protein engineering.

Background

10 Tissue plasminogen activator (tPA) is a key enzyme in a naturally occurring system by which mammals dissolve blood clots. Its value as a therapeutic agent, especially in the emergency treatment of coronary arterial or other vascular occlusions is widely recognized. tPA is a serine protease which catalyzes the conversion of the inactive protein plasminogen into plasmin, the
15 active form. Plasmin then dissolves the fibrin clot. tPA can bind specifically to fibrin molecules thus limiting its proteolytic action to the location of the clot. Other plasminogen activators presently available, urokinase, which is also derived from
20 mammalian sources, and streptokinase, which is derived from bacteria, do not exhibit this fibrin binding specificity. (Prourokinase however can bind to fibrin specifically.)

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Human tPA has been prepared in highly purified form from human melanoma cells by Rijken and Collen, J. Biochem. (1981) 256:7035-7041. Melanoma tPA (mtPA) is an approximately 68 kd protein which is highly glycosylated. Glycosylation is not believed to be essential to activity because enzymatic deglycosylation by endoglycosidase F does not reduce the activity of the protein, and tPA prepared from melanoma cells grown in the presence of the glycosylation inhibitor tunicamycin retains activity.

The complete amino acid sequence of tPA has been deduced from the cDNA sequence. See Pennica, D. et al, Nature (1983) 301:214-221. Recombinant forms of tPA have been produced in bacteria and in mammalian cells using appropriate control DNA sequences ligated to tPA cDNA (made from melanoma cell mRNA). The resulting protein product has the same amino acid sequence as the secreted protein from the melanoma cells.

20 Naturally occurring human tPA has the amino acid sequence and putative secondary structure shown in Figure 1. The figure also shows the signal sequences which are absent in the mature tPA protein. The sequence of the mature protein is considered to begin at position 1 designated in the figure and contains 527 amino acids.

The mature protein is thought to be composed of three major domains. The N-terminal 1-80 (approx) amino acids are designated the finger region. The sequence between approximately amino acid 80 and

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amino acid 270 is held in a double kringle con-
formation by six disulfide bonds. The kringle
region is putatively involved in the fibrin binding
properties of tPA. The portion of the molecule
5 responsible for the enzymatic action of tPA --the
catalytic domain-- is the region of amino acids
270-527 approximately. This portion of the molecule
appears to have the characteristics of a serine
protease.

10 Extensive studies have shown that various
regions of tPA are homologous to regions of other
proteins with related biological activities. For
example, kringle structures have also been found in
prothrombin, plasminogen and urokinase. The cata-
15 lytic domain has some homology to the bovine serine
proteases trypsin, chymotrypsin, and plasmin.

The tPA precursor zymogen is cleaved in vivo
between the arginine at position 275 and the iso-
leucine residue at 276. Cleavage results in con-
20 version of the zymogen to the active form of tPA.

Although the general parameters of production
of recombinant proteins from bacterial hosts are
understood, a number of practical problems are often
encountered. When mammalian proteins are produced
25 in prokaryotic host cells, often times either they
are unstable or they are precipitated within the
cell as "refractile" or "inclusion" bodies. In some
instances, these insoluble proteins are improperly
"folded", i.e. they are not processed by the host
30 cell into the appropriate three dimensional struc-
ture that is optimal or even adequate for activity.
Extensive efforts have been made to devise proce

dures to refold and to "reactivate" such proteins. See, for example, EPO publication 144,506, 1 August 1984, Genentech: U. S. Patent No. 4,511,502, Builder and Ogez. The tPA protein is especially susceptible
5 to improper folding because it contains 35 cysteine amino acid residues which are capable of forming disulfide bonds so as to stabilize whatever three dimensional structure, correct or incorrect, is generated in the expression system. Production of
10 foreign proteins in mammalian cells generally results in forms of the proteins which more accurately reflect their native conformations, but mammalian cell production is expensive and more difficult to control.

15 Disclosure of the Invention

This invention pertains to protein analogues of tissue plasminogen activator (tPA) which exhibit the proteolytic action of natural human tPA and optionally exhibit fibrin binding properties but are
20 generally molecules of lower molecular weight than natural tPA and are designed for highly efficient expression in prokaryotic expression systems. In general, the tPA analogues* can a) consist of

25 As used herein the term "tPA analogue" means a molecule other than tPA itself which comprises a catalytic fragment of tPA and which may include either an endogenous (tPA derived) or exogenous (non-tPA) polypeptide which may have fibrin binding activity.

substantially only a catalytic fragment of tPA; or
b) consist of a catalytic fragment of tPA linked to
a polypeptide which provides a desirable or enhanced
property or function in addition to the catalytic
5 function, provided that the polypeptide in combina-
tion with the catalytic fragment forms a molecule
which can be refolded efficiently. In general, the
analogue is of lower molecular weight than tPA.
Preferred polypeptides provide for one or more of
10 the following properties: i) fibrin binding ac-
tivity; ii) enhanced expression of the tPA analogue
in a particular expression system (either pro-
karyotic or eukaryotic); iii) stabilization of the
catalytic fragment in vivo; and iv) increased
15 biological half-life of the analogue in vivo.

Thus, in one embodiment, the tPA analogues of
this invention comprise essentially only a catalytic
fragment of tPA which contains the active enzyme
sites for proteolysis. Analogues of this type have
20 only the proteolytic properties of tPA. The pre-
ferred catalytic fragment is a fragment of human tPA
encompassing amino acids 262-527.

In other embodiments, the tPA analogues com-
prise a functional catalytic region and a second
25 region which provides any or all of the properties
described above. The catalytic fragment of such
analogues can be derived from the tPA molecule
catalytic. Fragments from other serine proteases
(urokinase, trypsin, etc.) could be used in similar
30 constructions.

The second region can be derived from tPA or nontPA proteins. A fibrin binding fragment, for example, can be derived either from natural fibrin binding domains of the tPA molecule or from exogenous polypeptides, proteins, or protein fragments which have fibrin binding properties. For instance, the fibrin binding fragment may be an endogenous portion of the tPA molecule involved in fibrin binding (e.g., parts of the finger/kringle region). Such fibrin binding regions can be modified for the purpose of enhancing expression efficiency. Portions of which might be tPA the finger/kringle region responsible for instability or suboptimal refolding of tPA as expressed in prokaryotic host cells can be excluded from the analogue to augment production of active analogues in prokaryotic systems.

Alternatively, a fibrin binding fragment of the analogues can be non-tPA fibrin binding polypeptides, i.e., exogenous polypeptide proteins or protein fragments which provide the analogue with fibrin binding capability and optionally other properties as enumerated above. The exogenous fragment can be of eukaryotic or prokaryotic origin. Examples of exogenous fibrin binding moiety of eukaryotic origin is the antigen binding domain (Fab) of an antifibrin immunoglobulin (Ig). Examples of prokaryotic fibrin binding polypeptides are the B domain of protein A, other domains of protein A, and fibrin binding domains of protein G.

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The analogues can also comprise fibrin binding fragments which are composites of prokaryotic and eukaryotic protein. For example, a composite comprising the B domain of Protein A and the tPA kringle region or portions thereof may be employed.

The tPA analogues are produced by recombinant DNA techniques. Although the analogues can be expressed in eukaryotic host cell systems, the preferred analogues designed for the more economical and convenient expression in prokaryotic host cell systems, e.g., E. coli. Thus, especially preferred tPA analogues are prokaryotic "leader" segments, such as the B domain of Protein A, which provide fibrin binding activity along with other advantageous properties. Indeed, tPA analogues comprising a tPA catalytic fragment linked to the B domain of Protein A have fibrinolytic activity and fibrin binding activity; they are efficiently expressed in prokaryotic host cells; and in comparison to natural tPA, they have higher solubility in physiological fluids and a longer half life in vivo.

Employing recombinant DNA techniques, the polydeoxynucleotides encoding the analogue are constructed and operably linked to control sequences of a suitable DNA expression vector. The recombinant vector is then used to transform the host cell. The transformed host cell is then cultured to produce the analogue which is then isolated from the host cell.

Brief Description of the Drawings

Figure 1 shows the primary and secondary structure of tPA including the 37 amino acid signal peptide.

5 Figure 2 (a and b) shows the complete DNA sequence of a preferred embodiment of the synthetic tPA gene.

Figure 3 is a schematic representation of the synthesis and ligation of the tPA 1 gene from
10 precursor oligonucleotides.

Figure 4 is a schematic for construction of expression vectors containing F_B -CF_{tPA} and F_B - F_B -CF_{tPA} gene constructs.

Figure 5 is a schematic for construction of
15 expression vector containing first kringle-CF_{tPA} gene construct.

Figure 6 shows the construction of a tPA analogue containing a hybrid kringle.

Figure 7 shows the nucleotide sequence of a DNA
20 construct encoding a tPA analogue comprising the B domain of Protein A linked to a catalytic fragment of tPA and the amino acid sequence of the analogue.

Figure 8 shows the results of a fibrin binding assay for F_B - F_B -CF_{tPA} analogue.

25 Figure 9 shows the binding of mtPA to fibrin.

Best Mode of Carrying Out the Invention

The tPA analogues of this invention comprise "recombinant" protein molecules which have the fibrinolytic activity of tPA and optionally the
30 capability to bind to fibrin. The molecules are

designed for highly efficient production in prokaryotic, especially E. coli. In preferred embodiments, the molecules are of lower molecular weight than natural tPA (one characteristic that provides for more efficient refolding and reactivation upon isolation from prokaryotic cells).

In one embodiment, the analogues comprise essentially only a catalytic fragment (designated CF) of the tPA or of another serine protease which has fibrinolytic activity (e.g. urokinase, streptokinase, trypsin, etc). The preferred catalytic fragment is the portion of natural human tPA encompassing amino acids 262-527 inclusive, i.e., the portion of the tPA molecule ranging from the serine/threonine residues 262 and 263 (which link the catalytic fragment to the second kringle of tPA) to the C-terminal proline residue (or substantial equivalents thereof). This preferred catalytic fragment can potentially form six intramolecular disulfide bonds which can aid in proper conformational folding of the catalytic fragment and stabilization of its structure (See Figure 1).

In another embodiment, the tPA analogues comprise the CF linked to a polypeptide, a protein or a protein fragment which helps stabilize the catalytic fragment, enhance expression of CF in a desired system and/or potentiate fibrinolytic activity of the catalytic fragment.

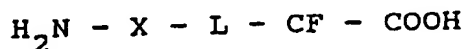
In the preferred embodiment, the tPA analogues have a fibrin binding capability. They comprise a catalytic fragment of tPA linked (either directly or through an intermediate polypeptide linker) to polypeptide protein or protein fragment which

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confers fibrin binding specificity. The polypeptide which provides fibrin binding specificity can be derived from "natural" tPA sequences involved in fibrin binding or exogenous amino acid sequences, either eukaryotic or prokaryotic, such as the antigen binding fragment (Fab) of an antifibrin antibody or the B domain of protein A.

In preferred embodiments, the catalytic fragment of tPA (designated CF_{tPA}) is linked to a polypeptide which confers i) fibrin binding capability; ii) increased solubility with respect to natural tPA in physiological fluids; iii) high expression in prokaryotic system; iv) increased biological half-life in vivo. The B domain of Protein A is an example of a prokaryotic polypeptide which when linked to CF_{tPA} provides a tPA analogue which has fibrinolytic and fibrin binding activity but is superior to natural tPA in that it can be expressed more efficiently in a prokaryotic host and has greater stability in physiological fluids and a longer half life in vivo.

In general, the tPA analogues of this invention can be represented by the formula:



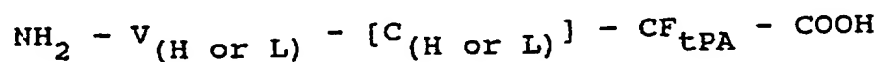
In this depiction, CF represents a catalytic fragment of tPA. The preferred catalytic fragment, as specified above, is the fragment CF_{tPA} encompassing

amino acids 262-527 of natural human tPA (or fragments having substantially equivalent catalytic activity). L taken together with X represents a bond (thereby defining a catalytic factor), or L individually represents a peptide bond linking a polypeptide, a protein or a protein fragment X to CF_{tPA} or L represents an oligopeptide linker of desired structure and function linking X to CF_{tPA} . It can be an amino acid sequence which provides a preferential site for cleavage. For example, L can be the cleavage site for Factor X_a . Incorporation of this site into the analogues provides for proteolytic cleavage of X and CF_{tPA} at the site.

The designation X represents a polypeptide, protein or protein fragment generally smaller in size than the complete finger/kringle region of tPA, linked (as a continuous polypeptide chain) to the catalytic fragment CF_{tPA} either directly or via an oligopeptide linker L. (In preferred embodiments, when L is present, the combined size of X and L is of lower molecular weight than the complete finger/kringle region of tPA). X can be a "leader sequence" which provides for a more stable and/or more efficiently expressed tPA analogue. Preferably, the polypeptide X is a fibrin binding polypeptide. Fibrin binding polypeptides may be selected from natural tPA fragments involved in fibrin binding. The selected fragments would have lower molecular weight than the combined finger/kringle region of tPA and accordingly the complete analogue would be of lower molecular weight than

tpA. The fibrin binding domain can be designed to exclude portions of the native molecule unnecessary for fibrin binding and/or portions which contribute to the instability or improper refolding of tPA.

- 5 X may be also selected from exogenous polypeptides sequences (those not found in tPA) of prokaryotic and/or eukaryotic origin which provide fibrin binding activity. An example of an exogenous eukaryotic protein fragment is the antigen binding domain of an immunoglobulin molecule which specifically bind fibrin. In analogues of this type, the variable region of an immunoglobulin light (V_L) or heavy chain (V_H) can be linked either directly or through a portion of the constant region of either chain to a catalytic fragment of tPA. This construct can be depicted as follows:



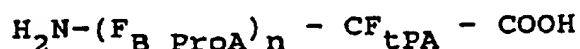
- wherein V is the variable region of a heavy (V_H) or a light (V_L) chain of a fibrin specific antibody and C represents a portion of the constant region of the antibody which is optional, as indicated by the brackets.

- Exogenous polypeptides may be chosen also from prokaryotic protein sequences which bind fibrin thereby providing a molecule comprising a prokaryotic "leader" linked to CF_{tPA} . A preferred prokaryotic polypeptide is the B domain of protein A which, surprisingly, has been found to provide fibrin binding specificity when joined to a catalytic

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fragment of tPA. In addition to providing fibrin binding capability, the B domain of protein A also provides for enhanced expression of the CF_{TPA} in E. coli; it provides a molecule with greater solubility (more easily recoverable from expression host) than natural tPA. It also provides for a longer biological half life in vivo. Because of this, the B domain represents an especially preferred prokaryotic polypeptide for construction of analogues of this invention. In addition to the B domain other domains of protein A, such as the B, C, or D domains, may also provide a fibrin binding capability as well as other desirable properties. These protein domains may be present in single or multiple units.

Analogues containing the B domain of protein A can be represented by the formula:



As above CF_{TPA} signifies a catalytic fragment of tPA, preferably the tPA catalytic fragment spanning amino acid residues 262-527 inclusive (or fragments having substantially equivalent catalytic activity).

F_{B ProA} designates the B domain of protein A or substantial equivalent thereof. Modified forms of the B domain can be used to enhance the specificity of fibrin binding and to reduce undesired immunogenicity of the segment. Multiple, sequentially aligned F_{B ProA} units may be attached to CF_{TPA} as indicated by the subscript n. Preferably, the

number of $F_B \text{ ProA}$ units may range from 1 to 5, most preferably, n is 1 or 2.

Analogues comprising the B domain of protein A linked to a catalytic fragment of tPA exhibit
5 fibrinolytic activity which is plasminogen dependent and which can be completely neutralized by antibody against tPA. These analogues bind fibrin directly. Further, $(F_B \text{ ProA})_n - CF_{\text{tPA}}$ analogues are less hydrophobic than natural tPA and, thus, may be
10 suitable for intramuscular routes of administration.

X may also be combinations (or composites) of eukaryotic and prokaryotic proteins. For example, X may be $F_B \text{ ProA}$ linked to a kringle region of tPA.

The tPA analogues of this invention are designed to be produced by recombinant DNA techniques.
15 The analogues can be expressed in prokaryotic host cell systems by standard techniques. Polydeoxynucleotides encoding the desired analogues can be constructed by conventional techniques of gene synthesis and gene splicing as set forth in more detail
20 below.

Double stranded DNA constructs encoding the tPA analogues can be represented, in 5' to 3' orientation, as follows:

25 5' [DNA encoding X] - DNA encoding CF_{tPA} 3' The brackets indicate that DNA encoding X is optional. Thus, the construct contains optionally a first DNA sequence encoding X and a second DNA sequence encoding CF_{tPA} . Where desired, a oligodeoxynucleotide encoding a linker oligopeptide L is interposed
30

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between the DNA sequence encoding X and the DNA sequence encoding CF_{tPA} . Nucleotides may be added to the 5' and 3' terminals of these constructs to provide restriction enzyme recognition sites for convenient insertion of the construct into DNA vectors.

When X is an exogenous, non-tPA polypeptide, DNA sequences encoding this portion of the analogue can be obtained from clonal cell lines harboring the DNA sequence. The cell lines can be obtained from commercial sources and the DNA sequence can be obtained by standard techniques of gene cloning. When the DNA sequences encoding the desired polypeptide X are known (as is the case for the B domain of protein A) or can be determined (sequenced), the polydeoxynucleotide can be synthesized by standard procedures such as the phosphotriester and phosphite triester techniques. DNA encoding the tPA derived fragments, e.g., CF_{tPA} , can be obtained from DNA sequences encoding tPA. As an example of a complete DNA construct, Figure 5 shows the entire deoxynucleotide sequence of a gene construct encoding the analogue: $F_B \text{ ProA} - CF_{tPA}$. The amino acid sequence of the analogue is also given.

In the preferred embodiments, DNA sequences encoding the tPA-derived portions of the analogues are obtained from a synthetic tPA gene. For this purpose, applicants have synthesized a conveniently manipulable, synthetic tPA gene which encodes human tPA. The synthetic gene has been designed to be compatible with the E. coli codon bias for efficient expression. The synthetic DNA fragment encodes a

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protein having tPA activity and contains inherent characteristics which permit facile manipulation of subgenic DNA fragments. The preferred gene encodes a protein with an N-terminal serine (after the methionine corresponding to the start codon) although a
5 useful variant is a fragment which contains three additional N-terminal amino acids, Gly-Ala-Arg, which precede the N-terminal serine exhibited by most native mature tPA, and which are sometimes
10 retained by the mature sequence in the extracted protein.

The gene is bounded by additional short segments of DNA if necessary to provide start and stop codons for translation and to provide convenient
15 restriction sites. Thus, the gene will contain approximately 3×527 , or 1581 base pairs, with a few additional base pairs at each terminus.

The preferred tPA gene construct is shown in Figure 2. It is prepared as set forth below in
20 Example 1. The synthetic gene bears an additional 9 base pairs including the ATG start codon at the upstream end, and an additional 2 base pairs to complete the stop codon at the downstream terminus. By appropriate codon selection, the fragment is
25 bounded by EcoRI and Bam HI restriction sites. Additional restriction sites were designed into the molecule as shown in Figure 2.

The particular sequence illustrated in Figure 2 was designed using the criteria of compatibility
30 with E. coli codon preference, the proper distribution of restriction enzyme sites across the gene,

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and the uniqueness of these sites within particular segments representing the whole. It is clear that alternate DNA fragments which encode substantially the entire tPA protein with different restriction sites could be constructed using different codon selection. The preferred design, however, permits easy segregation of the DNA into three "cassettes", each cassette corresponding to approximately a third of the molecule. By using this cassette-based construction, the resulting protein can be readily manipulated at the genetic level to represent an assembly of the most desirable portions in an optimal order.

In addition, during construction, restriction sites unique to individual cassette are included, thus permitting clean excision of shorter portions of the molecule. The sub-cassette fragments can be excised and rearranged to provide DNA constructs encoding a desired tPA fragment.

The genetic constructs encoding the tPA analogues can be assembled and expressed by conventional recombinant DNA techniques. In general, DNA sequences are cleaved using restriction enzymes which are commercially available. The conditions of cleavage as to pH, time, temperature, concentration of enzyme are typically specified by the manufacturer. After each incubation, the enzyme is removed by extraction, for example, with phenol/chloroform and the nucleic acid fraction is recovered by precipitation with ethanol. Size separation of the cleaved fragments may be performed

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using standard agarose or polyacrylamide gel electrophoresis techniques as described in Methods in Enzymology (1980) 65:499-560. Fragments may be blunted if desired by treating with E. coli DNA polymerase I (klenow fragment) in the presence of the four deoxynucleotide triphosphates (dNTPs) using ambient temperature for about 30 minutes in 50mM Tris.HCl pH 7.6, 50 mM NaCl, 6 mM MgCl₂, 6 mM DDT and 5-10 uM of the dNTPs. The extent of fill-in at the sticky ends may be, of course, regulated by appropriate choice of the dNTPs. Treatment under appropriate conditions with S1 nuclease removes single stranded portions.

Ligations are performed using T4-DNA ligase at pH 7.5 in Tris buffer, under conditions recommended by the manufacturer.

Construction of correct DNA sequences is confirmed by transforming E.coli or other suitable host, selecting successful transformants using the appropriate antibiotic resistance or other markers, and isolating plasmids from transformants, for example, by the method of Clewell, D., et al, Proc Natl Acad Sci (USA) (1969) 62:1159. Isolated DNA is analyzed by restriction enzyme mapping and/or sequencing. Sequencing uses either the dideoxy method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463, as further described by Messing, et al, Nucleic Acids Res (1981) 9:309 or by method of Maxam, et al, Methods in Enzymology (1980) 65:499.

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Transformation of DNA vectors into E. coli or other prokaryotes is performed as described by Cohen, S.N., Proc Natl Acad Sci (USA) (1972) 69:110; for mammalian cells transformations are by the method of Graham and Van der Eb. Virology (1978) 52:546.

Alternatives and modifications of the foregoing methods are also employable, but the methods outlined above typify those useful in the construction of the DNA sequences encoding the tPA analogues and the synthetic tPA gene.

The DNA constructs encoding the tPA analogues can be expressed in prokaryotic expression systems. The preferred prokaryotic host is E. coli, although other bacterial strains such as Bacillus, Pseudomonas, or other Gram-positive or Gram-negative bacteria can also be used. A DNA construct encoding the desired tPA analogue is linked to control systems compatible with the host and disposed on a suitable DNA expression vector which is capable of replication in the host cell. The DNA expression vector comprises, in order, a promoter of bacterial or phage origin, a ribosome binding site, a translational initiation signal, the DNA construct encoding the tPA analog (see above), a translation termination site, and a terminator. Preferably, the vector also carries a gene encoding a selectable marker.

Common plasmid vectors include those derived from pBR322 and the pUC series. Charon 4 lambda phage is a frequently employed phage vector.

Control sequences include promoter and ribosome binding site encoding sequences. A variety of such control sequences are available, most commonly the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et. al. Nature (1977) 198:106) and the tryptophan (trp) promoter system (Goeddel, et. al, Nucleic Acids Research (1980) 8:4057). Composite promoters containing elements of both the trp and lac promoter systems are also available. For the expression of F_B -CF and F_B - F_B -CF constructs the trp and tac promoters are preferred.

Eukaryotic microbes may also be used for expression of the analogues, most commonly laboratory strains of Saccharomyces cerevisiae, or Baker's yeast. A number of yeast control systems and vectors are available, including those which are promoters for the synthesis of glycolytic enzymes (Hess, et al. J. Adv. Enzyme Req. (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Yeast vectors employing the 2 micron origin of replication are suitable as transfer vectors (see, for example, Broach, J.R., Methods Enzymol. (1983) 101:307).

Tissue culture cell lines immortalized from mammalian or other higher organisms can also been used as recombinant hosts. Such cell lines include Chinese hamster ovary (CHO), Vero, HeLa, and Cos cells. In general, the Cos cell system is used for transient expression, while CHO cells typically integrate transformed DNA into the chromosome. Suitable mammalian vectors are generally based on viral origins of replication and control sequences

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Most commonly used are the simian virus 40 (SV40) promoters and replicon (Fiers, et al, Nature (1978) 273:113) and similar systems derived from Adenovirus 2, bovine papilloma virus, or avian sarcoma virus.

5 The tPA analogues expressed as heterologous protein in host cell systems can be purified and renatured by standard techniques for isolation and renaturation. Alternatively, an improved technique for isolation and renaturation of protein from E.
10 coli cells as set forth below (and in further detail in the Exemplification), can be used. According to this technique, E. coli cells containing heterologous protein (tPA analogue) are lysed in a standard buffer and the cellular components are sedimented
15 by, for example, centrifugation. The cells are resuspended and washed in a buffer containing 20-40% (preferably 25%) sucrose. The cells are then washed in a buffer containing 20-40% sucrose and a detergent such as deoxycholate. After the washes, the
20 cells are sedimented, separated from the wash solution, resuspended in buffer and lysed in the presence of a protease inhibitor. Insoluble, heterologous protein (generally present in the form of inclusion bodies) is solubilized by extraction
25 with a buffer containing a strong denaturant such as 2-8M urea (preferably 4M urea) or 6-8M guanidine. HCl. The protein is subjected to sulphitolysis and then renatured in a buffer containing reduced and oxidized glutathione in approximately a 10 to 1
30 molar ratio. The renatured protein can then be

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dialyzed to remove the denaturant. The renatured tPA analogue can further be purified by chromatographic steps such as gel filtration and/or affinity chromatography.

- 5 In addition to production by genetic engineering techniques, the tPA analogues can be synthesized by procedures of chemical protein synthesis such as the solid phase procedure of Merrifield.

10 The invention is illustrated further by the following exemplification.

Exemplification

I. Synthesis of tPA gene

15 The entire coding sequence for mature human tPA (shown in Figure 2) was synthesized from three approximately equal-length gene fragments designated tPA1 (base pairs encoding the initiation site and amino acids 1-173), tPA2 (base pairs encoding and amino acids 174-346), and tPA3 (base pairs encoding amino acids 347-527 and stop codon). Each of these
20 gene fragments was, in turn, constructed from oligonucleotide subunits. tPA1 required the synthesis of 70 oligonucleotides de novo: the entire tPA gene required the synthesis of 215 oligonucleotides.

25 a. Chemical Synthesis of Oligonucleotides

 The chemical synthesis of the 215 oligonucleotides was accomplished by two methods: phosphotriester (ca. 50% of the fragments) (Crea and Horn, Nucl. Acids Res., 8, 2331, 1980) and phosphite

triester method (Alvarado-Urbina et al., Science 214, 270, 1981).

Described below is a typical oligonucleotide synthesis by the phosphite method, involving the use of automatic synthesizer and the "in situ" activation of the phosphite nucleotide intermediates. The phosphite method is the method of choice, being suitable to automation.

Materials and Methods

10 Anhydrous acetonitrile and 2,6 lutidine were prepared by distillation from calcium hydride and stored over activated molecular sieves. The 5'-DMT-N-protected deoxyribonucleosides were prepared by standard methods and lyophilized from 1,4 dioxane prior to use. Chloro-diisopropylaminomethoxy phosphine was purchased from American Bionuclear.

In situ preparation of phosphoramidites

In a typical preparation, an oven-dried septum bottle was charged with 10ml of anhydrous acetonitrile, 0.6ml of 2,6 lutidine and 0.2ml of chloro-N, N-diisopropylamino phosphine. A 1 mmol portion of lyophilized 5'-DMT-N-protected deoxyribonucleoside was dissolved in 5ml of anhydrous acetonitrile and injected into the reaction vessel. The nucleoside vial was washed with an additional 5 ml of acetonitrile and the wash solution was injected into the reaction vessel. The reaction mixture was stirred for 10 minutes at ambient temperature and activated by the addition of a solution containing 210mg of 1H-tetrazole in 30ml of anhydrous acetonitrile. Phosphoramidite solutions prepared in this manner

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have been used over time periods exceeding 12 hours with satisfactory results.

Oligonucleotide Synthesis

5 Oligonucleotide synthesis was carried out on a silica solid support using the automated synthesizer described below. A standard cycle consisted of the following 10 min, stop-flow protocol:

	<u>Reagent</u>	
	Phosphoramidite	01:00
10	Stop Flow	01:00
	Oxidation*	00:30
	Pyridine Wash*	01:30
	Methylene Chloride Wash	01:30
	DMT Deblock**	01:30
15	Methylene Chloride Wash	01:00
	Acetonitrile Wash***	02:00

Flow rate = 5ml/min

* 1% I₂ in 3:1:1 THF:pyridine:H₂O

** 3% Trichloroacetic acid in CH₂Cl₂

20 *** Dried 24 hr in over activated molecular sieve

Automated Synthesizer

The instrument consisted of a series of pneumatic three-way valves, 120v solenoids and a pump. Sole-noid operation was effected via BSR-X10
25 switches under control of a Commodore 64 computer equipped with a VIC controller (Genesis Computer Corporation). The minimum configuration required by

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the protocol described above would utilize 10 valves, 5 solenoid units and 10 BSR switches. We have enhanced the capability of the instrument by the addition of valves to provide for on-line catalyst mixing, recycling of reagents, collection of DMT solution during deblocking and multicolumn sequential synthesis.

The control software was written in BASIC and utilized a machine language subroutine to effect valve operation. The menu-driven software allowed for creation and storage of various synthesis protocols, completely automated synthesis, and full manual override of all functions. Protocols were created in response to machine prompts and allowed the use of phosphotriester or phosphite methods as well as on-column phosphitylation of the growing oligonucleotide. Additionally, the ability to run in a stop-flow mode allowed the use of smaller quantities reagents than required in continuous flow operation. The synthesis subroutine prompts the use to input the desired protocol, sequence, and other data required for printout of production records. At any time during the synthesis, the user may escape to the manual override subroutine, operate any of the valves and return to the synthesis.

Workup and Purification

Oligonucleotide cleavage and deprotection was effected by the standard two-step procedure consisting of treatment with 2:1:1 dioxane/triethylamine/thiophenol, followed by overnight treatment with

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concentrated ammonia at 55°C. The resulting solution was evaporated to dryness and dissolved in 1ml of 3:1 water/ethanol.

- Purification was carried out by streaking
- 5 0.15ml of the crude oligonucleotide solution on a 20x20 cm Kieselgel 60 TLC plate and eluting with 5.75:3.25:10 n-propanol/concentrated ammonia/water. The product band was visualized under UV, scraped
- 10 from the TLC plate and eluted with 3:1 water/ethanol. The purified oligonucleotides were checked for the correct size homogeneity by gel electrophoresis of 0.05 OD sample on polyacrylamide gel with .5X TBE buffer and 7M urea. The gel was stained with ethidium bromide and visualized under UV light.
- 15 Following phosphorylation with T4 kinase, all possible pairwise ligations were tested at 37°C for 30 min. Positive results from these ligation tests were taken as partial confirmation of sequence pending sequence analysis of the assembled gene.
- 20 Synthesis, purification and quality control was similar for all 215 oligonucleotides. Fragments that failed to pass purity and size tests were resynthesized and purified as above.

b. Gene Ligation

- 25 The three genes, tPAI, II, and III were obtained by the enzymatic ligation (DNA-ligase) of oligonucleotides, each using a similar ligation approach. The general scheme of tPAI gene ligation is shown in Figure 3.

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The tPA I gene was constructed from 70 oligonucleotides. Eight large gene fragments, I to VIII, were synthesized from oligonucleotides by 5' phosphorylation followed by enzymatic ligation. Phosphorylation was carried out at 37°C for 1 hr in a reaction mixture containing oligonucleotides (1.2 ug of each), 1mM ATP, T4 phosphorylase kinase (1.3 units/ug DNA), 70mM Tris.HCl, pH 7.6, 10mM MgCl₂, and 5mM dithiothreitol. Ligation of the phosphorylated oligonucleotides was carried out at 15°C for 2 hr in a reaction mixture containing 0.075 mM ATP, T4 DNA ligase (1.5 unit/ug DNA), 50mM Tris.HCl, pH 7.8, 10mM MgCl₂, 20mM dithiothreitol, and 50 ug/ml BSA. The DNA fragments were resolved by electrophoresis on 8% polyacrylamide gels in the Tris-borate-EDTA buffer system described by Maniatis et al. (Proc. Natl. Acad. Sci. USA, 72,1184, 1975). Bands migrating at the expected molecular weight were sliced from the gel and were electroeluted using published techniques (Maniatis et al., "Molecular Cloning", Cold Spring Harbor Laboratory, N.Y., 1982). The eluted DNA was dried under vacuum and was resuspended in 200 ul of 0.2M sodium acetate, pH 5. The sample was extracted twice with equal volumes of phenol, chloroform, and isoamyl alcohol (50:50:1), and once with chloroform. The DNA was precipitated with 2.5 volumes of absolute ethanol. The purified gene fragments were stored at 4°C in 1mM Tris.HCl, pH 7.5, and 0.1mM EDTA.

Stoichiometric amounts of gene fragments I to VIII (5.4 ug total) were combined and ligated using

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the conditions described above. After gel electrophoresis, the DNA band that migrated at 530 base pairs was sliced from the gel, electroeluted, and purified as described above.

5 c. Molecular Cloning

The synthetic tPAI gene was inserted into the EcoRI and BamHI sites of pUC8 (Viera and Messing, Gene, 19, 259, 1982). pUC8 (6 ug) was digested at 37°C in a 40 ul reaction mixture containing BamHI
10 (32 units), 6mM Tris.HCl, pH 7.9, 6mM MgCl₂, 150mM NaCl, and 3mg/ml BSA. After one hour, 6 ul of 1M Tris.HCl, pH 7.5, and 40 units of EcoRI were added to the digestion mixture. The volume was adjusted to 60 ul with sterile water, and the digestion was
15 allowed to proceed at 37°C for an additional hour. The DNA fragments were resolved by electrophoresis on a 6% polyacrylamide gel. The large fragment was sliced from the gel and was electroeluted. The synthetic tPA in gene (30ng) and the large EcoRI/
20 BamHI fragment of pUC8 (100ng) were combined and treated with T4 DNA ligase. The ligation mixture was used to transform competent E. coli K12 JM83 cells. Competent cells were prepared by using the calcium chloride method described by Maniatis et al.
25 supra. Transformants were selected by plating on LB agar (Maniatis et al., supra) containing 50 ug/ml ampicillin. Plasmids were isolated from small cultures of transformed bacteria by using a modification of the method of Birnboim and Doly (Nucl.
30 Acids Res., 7, 1513, 1979) as described by Maniatis

et al., supra. Purified plasmids were screened for the presence of the 530 base pair tPAI gene insert by polyacrylamide gel electrophoresis after digestion with EcoRI and BamHI. Large scale preparations of plasmids containing the tPAI gene insert were carried out by using the alkaline lysis method of Birnboim and Doly, supra.

d. DNA Sequence Analyses

The DNA sequence of the cloned tPAI gene was determined by the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci. USA, 74, 5463, 1977). pUC8 plasmid containing the gene was cleaved with EcoRI and BamHI, and the gene insert was purified by gel electrophoresis. The tPAI gene was inserted into the EcoRI and BamHI sites of M13 mp 18 and mp 19 (Messing, supra). Single stranded M13 templates were prepared by using the method of Schreier and Cortese (J. Mol. Biol., 129, 169, 1979).

e. Verification of the Synthetic Gene Sequences

The sequencing of the synthetic gene was done on the 6 fragments that were used as building blocks of the tPA gene; thus, errors were eliminated before the whole molecule was completely assembled. A second sequencing analysis was performed after completion of the construction in order to assure that no deletions had occurred during the multiple steps of assembly. While the shorter fragments can

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be directly sequenced after cloning into the master cloning sites of the m13 phage genome using the m13 oligonucleotide primer to initiate the polymerase reaction, the whole gene is too large to be sequenced in one reaction. Therefore, internal primers were used that had been chosen from oligonucleotides of tPA retained from the synthesis of the gene and spaced at appropriate intervals. These intervals were approximately 250bps which allowed sufficient overlapping of the sequences.

In addition to the sequencing analysis, the reading frame of the initial fragments and combinations of adjacent fragments were tested by direct expression in E. coli and the immunoreactivity of the resulting polypeptides was analyzed. While single base deletions could potentially be overlooked during sequencing they would cause a change in reading frame for the translation of protein. Consequently, an alteration in polypeptide size in conjunction with a loss of antigenic determinants would be observed. These tests provided valuable insights into the expression behavior of various domains of the tPA molecule and confirmed the reactivities of the antisera being used.

25 II. Expression of tPA Analogues in E. coli

Construction of Expression Vectors

The construction of the plasmid for the direct expression of catalytic fragment was facilitated by the ligation of a ScaI to BamHI fragment from pF80,

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a plasmid which contained the entire tPA gene, and a synthetic EcoRI to ScaI DNA to a cut pUC plasmid to form pI46. The resultant CF gene, an EcoRI to BamHI fragment was inserted into a pKK (obtained from Pharmacia; see J. Brosius, PNAS 81 6929, 1984) expression vector to form pI97. The catalytic fragment was directly expressed under the control of the tac promoter (Figure 4). To synthesize the F_B -CF fusion protein, an EcoRI to NarI fragment, containing the F_B nucleotide sequences and isolated from pH116, (a pUC vector containing F_b sequence) and a synthetic NarI to ScaI linker were inserted into pI97 cut with the restriction enzymes, EcoRI and ScaI (Figure 4) to form pH128. The use of the synthetic linker deleted the initiation codon and joined the F_B polypeptide directly to the first amino acid (ser) of the catalytic fragment. The F_B dimer catalytic fragment (F_B - F_B -CF) was constructed by opening the F_B -CF gene at the unique MluI site in the F_B coding region and inserting an MluI fragment derived from a dimer of F_B coding sequences (Figure 4) to form pI100. To construct for the expression of F_B - X_a -CF, a synthetic NarI to ScaI DNA fragment was inserted into pI46, which was a plasmid containing the gene for the catalytic fragment, to form pP1. Both the NarI to BamHI fragment, isolated from pP1, and the EcoRI to NarI fragment, containing the F_B coding sequences from pH116, were ligated into pI46, opened at both the EcoRI and BamHI sites to form pP4. To express the F_B - X_a -CF, the complete gene was isolated in two DNA fragments: EcoRI to SacI and ScaI to PstI nucleotide sequences. These two fragments were

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inserted into a pKK expression vector as pP5 under the control of the tac promoter.

To construct the tPA analogue containing the hybrid kringle, the NarI to NarI fragment in the two
5 kringle regions was deleted (Figure 5). In order to delete this fragment, two DNA fragments: a EcoRI to NarI and a NarI to BamHI nucleotide sequences, were ligated into pUC8, digested by restriction enzymes, EcoRI and BamHI. The resultant gene was then
10 isolated in two pieces: a EcoRI to HindIII fragment and a HindIII to PstI fragment, and inserted into the expression vector to fuse with the trp leader by a three-factor ligation.

The tPA analogue containing only one kringle
15 region was constructed by deleting the second kringle (Figure 6). The vector was pUC with tPA 123, cut at SphI and BamHI, dropping out tPA2 and tPA3. A fragment containing part of tPA2 was isolated between PstI (at 789 bps) and BamHI (at
20 1051 bps). These fragments were ligated after addition of two strands of synthetic DNA, CBM # 2014 and CBM # 2015; synthetic spH1 to Pst1 linker). The resulting construct was deficient of the tPA domain.

In the next construction step, the single
25 kringle analog was completed by combining in pUC a fragment, bearing only the first kringle, spanning from EcoRI (at 1 bps) to AvaI (formerly at 1042 bps), and another fragment containing tPA3 from AvaI (1042 bps, partial digest) to SalI (1599 bps).

30 Other construction (including the use of the entire tPA gene) were made and expressed. The level

of expression varied depending on the nature of the protein expressed.

Competent E. coli K12 RB791 (obtained from Jurgen Vrosius, see, PNAS 81 6929-6933, 1984) were
5 mixed on ice with 50 ng of the tPA encoding plasmids for 30 min followed by heat shock at 40°C for 2-3 min and then grown on 1 ml LB medium without antibiotics for 30 min. The cells were spun down and resuspended in 100 ul LB medium, and then trans-
10 ferred to agar containing 20 ug/ml tetracycline. Resistant colonies were picked, grown in LB medium, and induced by the addition of 10 g/ml indole acrylic acid (IAA).

To assay for the expression of protein, the
15 cells were harvested 5-10 hours after induction and the cell paste was dissolved in 6 M guanidine.HCl containing 25 mM Tris.HCl buffer, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and sonicated briefly. The sonicate was dialyzed vs the same buffer without guani-
20 dine. Aprotinin (10 units/ml) was added during during dialysis to protect the protein from E. coli serine proteases.

To assess biological activity, a fibrinolytic assay was conducted on each crude extract before and
25 after dialysis; cells without DNA encoding the catalytic region of tPA showed no fibrinolytic activity. Thus, fibrinolysis could not be attributed to nonspecific proteases in the E. coli extract.

Preparation of Inoculum for Bacterial Fermentation

A frozen E. coli stock containing the desired plasmid was inoculated into 200 ml of LB broth,

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containing 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl and 1 ml/l of a tetracycline stock (10 mg/ml of 95% ethanol and 5% isopropanol) in a 1 liter baffled shaker flask. The culture was incubated on a rotating platform at 200 rpm for 17 hours at 37 C.

Fermentation of E. coli

The overnight culture was inoculated into 10 liters of 1 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 8 g/l glucose, 5 g/l caesin hydrolysate, 5 g/l NaCl, 3 g/l KH_2PO_4 , 1 g/l NH_4Cl , 1 ml/l 1 M MgSO_4 , 0.5 ml/l 1% tryptophan in glacial acetic acid, 0.1 ml/l 1M CaCl_2 , 1.5 ml/l of a trace mineral mix (27 g/l $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.3 g/l ZnCl_2 , 2 g/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2 g/l $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.3 g/l $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$), 0.5 g/l H_3BO_3 and 100 ml of concentrated HCl) and 2 ml of 10 mg/ml tetracycline, pH 7.0 in a 14 liter fermentor. The bacterial culture was agitated at 300 rpm and incubated at 37 C. The pH of the culture medium was maintained in the range of 6.85 to 7.15 by the addition of either a solution of 10% phosphoric acid or a stock of 1M NH_4OH . The culture was sparged with air at a flow rate of 10 liter per min.

When the desired cell density for the bacterial culture containing an expression vector under the control of the tac promoter was reached, isopropyl-thiogalactopyranoside was added to a final concentration of 0.1 mM. After induction, the pH controller was turned off and the fermentation was

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allowed to proceed until the pH of the medium increased to 7.4.

Cell Recovery

At the end of the fermentation, 10 liters of the bacterial culture were decanted into a 20-liter carboy. An Amicon hollow fiber ultrafiltration unit was used to concentrate the culture medium down to 1.5 l. The bacteria were pelleted in 500 ml bottles by centrifugation at 6000 rpm for 10 min. After decanting the supernatant, the cell pellet was transferred to 50 ml capped tubes and stored at -70 C until use.

Preparation of inclusion bodies from E. coli.

After fermentation, 1 gm of wet cell paste was suspended in 10 ml of 25% sucrose in 25 mM Tris.HCl, 10 mM EDTA, 0.1 M NaCl, pH 8.0. The cells were incubated on ice for 10 minutes and the cell suspension was centrifuged at 8000 rpm for 10 min at 4 C in a Beckman refrigerated J21-B centrifuge. After decanting the supernatant, the cell pellet was resuspended in 10 ml of 25% sucrose in 25 mM tris-HCl, 10 mM EDTA, pH 8.0. Addition of 5% of detergent to the wash resulted in removal of a greater number of contaminating proteins from the inclusion bodies. After incubation on ice for 10 minutes, the cells were centrifuged at 8000 rpm for 10 minutes. After removing the supernatant, the cell pellet was resuspended in 10 ml of 25 mM Tris.HCl, pH 8.0 and again incubated on ice for 10 minutes. 20 KIU per

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ml of aprotinin or 0.5 mM PMSF was added to the cell preparation to inhibit protease. To lyse the cells, 1 mg of lysozyme was added to the cell suspension and mixed by vortexing. After incubation on ice for 5 minutes the cells were broken by sonication, 3 x 1 minute bursts separated by 1 minute intervals. Then, the inclusion bodies and cell debris were pelleted at 17,000 rpm for 20 minutes at 4 C. The inclusion body preparation was be stored at -80 C or used immediately.

Solubilization of protein

Protein can be sequentially extracted from the inclusion body preparation by resuspension into 10ml of 25 mM Tris.HCl, 10 mM EDTA, pH 8.0; 10 ml of 25 mM Tris.HCl, 10 mM EDTA, containing 1% Triton X-100 or 0.5% sodium deoxycholate; 10 ml of 25 mM Tris.HCl, 10 mM EDTA, pH 8.0, containing 2 to 4 M urea; 10 ml of 25 mM Tris.HCl; 10 mM EDTA, pH 8.0, containing 6 to 8 M guanidine.HCl; and finally 10 ml of 25 mM Tris.HCl, 10 mM EDTA, 6-8 M guanidine.HCl, 1% B-mercaptoethanol, pH 8.0.

Sulfitolysis and renaturation

Protein solution was diluted into 6 to 7 M guanidine.HCl, 25 mM Tris.HCl, 10 mM EDTA, pH 8.0, containing 20 mg/ml sodium sulphite and 10 mg/ml sodium tetrathionate. The optimal protein concentration for sulfitolysis was determined to be 125 ug/ml. The protein solution was incubated at room temperature for at least 15 hours. Then, the

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protein was diluted into a renaturation buffer consisting of 25 mM Tris.HCl, 10 mM EDTA, 0.01% tween, 1 M guanidine.HCl, 1 mM reduced glutathione and 0.1 mM oxidized glutathione, pH 8.3 to 8.5. The
5 renaturation of protein was allowed to proceed at 22 C for 7 to 24 hours. After renaturation, the guanidine-HCl has to be removed immediately by dialysis to retain the biological activity.

Purification of the refolded catalytic fragments

10 Both F_B -CF and F_B - F_B -CF were partially purified by immunoaffinity chromatography on a PAM-2 column (American Diagnostica, Conn). PAM-2 is a monoclonal antibody specific for two-chain m-tPA. 10 mg of
15 antibody were coupled to 1.5 ml of CNBr activated sepharose 4B. About 1 to 2 mg of refolded catalytic fragments were allowed to pass through the column at a flow rate of less than 30 ml per hour at room temperature. Adsorption of the catalytic fragments
20 to the immobilized antibody could also be facilitated by incubating the protein with the beads overnight at 4 C on a rotating platform. The column was then washed with 10 bed volume of 20 mM Tris.HCl, 0.1 M NaCl, pH 7.5. The bound protein was then
25 eluted sequentially with 0.25 M KSCN and 1.5 M KSCN in 20 mM Tris.HCl, pH 7.5. Protein elution profile was monitored by a chromogenic assay using substrate S-2251 before or after extensive dialysis against 0.5 mM Tris.HCl, pH 7.5 at 4 C. After dialysis, the

protein concentration was determined by Bio-rad protein assay using bovine serum albumin as a standard.

Biological Assay of tPA and catalytic fragments

5 Fibrin plate assay

A 0.5% plasminogen-rich bovine fibrinogen solution was prepared by dissolving the fibrinogen powder (75% clottable, Miles Scientific) in 50 mM Tris.HCl, 90 mM NaCl, 0.01% sodium azide (optional),
10 pH 7.5, at 37 C. The fibrinogen was then cooled to 4C by incubation on ice. To 10 ml of the cooled solution, 0.18 ml of a 20 U/ml bovine thrombin solution (CalBiochem) was added. Immediately after the thrombin was thoroughly mixed with the fibrinogen,
15 the solution was poured onto a assay plate. The fibrinogen was allowed to clot at room temperature for about 30 minutes. To assay for the biological activity of the catalytic fragments, 10 ul of sample after diluted 1:1 with 0.25% swine gelatine
20 (Sigma) in 50 mM Tris.HCl, 90 mM NaCl and 0.1% Tween 80. The plate was incubated at 37 C overnight. Relative biological activity of the samples corresponds to the diameter of the lytic zone around the sample. Urokinase or m-tPA served as the reference.
25 The sensitivity of the assay is in the range of 0.62 to 20 U/ml.

Chromogenic assay

About 1 to 4 ul of the sample was mixed with 30 ul of 10 mM Tris.HCl, 0.1% Tween 80, 12.5 munits of

plasminogen, 0.78 mg plasminogen-free fibrinogen, pH 7.5, and incubated in a 37 C water bath for 10 minutes in a 96-well microtiter plate directly or in 1.5 ml Eppendorf tubes. Then, 70 ul of 0.5 mM chromogenic substrate, S-2251 (Kabi Vatrium, Sweden) were added to the reaction mixture followed by incubation for another 30 minutes. The reaction was stopped by the addition of 10 ul of 50% glacial acetic acid. The color developed was recorded in a plate-recorder at A405. Melanoma-tpA (American Diagnostica) calibrated by the international standard from the National Bureau of Standard (London, UK) was used as a standard. The sensitivity of the assay is in the range of 5 to 25 u/ml.

15 Binding of F_B - F_B -CF analogue to fibrin

Wells were coated with fibrinogen or bovine serum albumin (BSA) by drying 200 ul of a 10 mg/ml solution in a 96-well plate. The fibrinogen was then converted into fibrin by incubation with 4 munits of thrombin in 100 ul of phosphate buffered saline, pH 7.2 (PBS) at 37 C for 1 hour. Various quantities of F_B - F_B -CF, as indicated in Figure 6, were then added to the well in 100 ul PBS and incubated at 37 C for 2 hours to facilitate binding. After incubation, the supernatant was removed and the wells were then washed once with 100 ul PBS. The amount of bound and unbound F_B - F_B -CF were determined by chromogenic assay.

Molecular weight determination by zymographic analysis

The molecular weights of the active catalytic fragments were estimated by zymographic analysis.

5 An 11% SDS polyacrylamide gel containing 0.1% gelatin and 1.5 units of plasminogen was prepared. Electrophoresis was allowed to proceed at 4 C for 3 to 4 hours, at 10 mamp per gel. Then, the gel was incubated with shaking at room temperature in 2.5% triton X-100 to remove the SDS in the gel so as to

10 restore the biological activity of the catalytic fragments. The gel was then incubated in 0.1 M glycine.HCl, pH 8.3 at 37°C for 3 hours. After the incubation, the gel was stained with Bio-rad protein

15 reagent diluted 1:8 with deionized H₂O.

RESULTS

Preparation of inclusion bodies

The first wash of the cells with hypertonic solution (25% sucrose) collapsed osmotically the

20 inner membrane, thereby releasing a large number of cellular proteins, especially when the expression level of the catalytic fragments was high. The choice of detergent used in the second wash depended on the solubility of the catalytic fragment. Cells

25 expressing the trp-CF, the least soluble catalytic fragment, were washed with 1% dodecyl sulphate without solubilizing the inclusion bodies. However, the protein in the inclusion bodies could be solubilized under the same conditions when they are

purified from the cells. Cells expressing other catalytic fragments cannot withstand this treatment. A weaker detergent, such as 1% Zwittergent 3-16 (Z-16) or deoxycholate was used without solubilization of the inclusion bodies before lysis of the cells. Deoxycholate has an added advantage over the other detergent in that it is dialyzable. The prewash with detergent before lysis of the cells removed considerable amounts of contaminating proteins.

Solubilization of catalytic fragments

Various analogues of catalytic fragments exhibit differences in solubility. Of all the catalytic fragments studied, the solubility in aqueous buffer decreases in the order of F_B-F_B-CF , F_B-CF , CF , $trp-CE$. The varying degree of solubility can be attributed to the leader sequences of the molecules. Of the two denaturants used to recover protein from inclusion bodies, i.e., urea or guanidine.HCl, both proved to be satisfactory. In the case of urea extraction, 4M was determined to be the optimal concentration. 2 M urea did not result in recovery of all the desired protein; while extraction with 8 M urea resulted in a high degree of contamination with the other cellular proteins. After extraction, the urea was removed completely by dialysis against Tris.HCl buffer without precipitating any proteins. However, when proteins were extracted with 6-8 M guanidine.HCl, precipitation of

proteins occurred when the guanidine.HCl was removed directed by dialysis.

Renaturation of the catalytic fragment

5 To reduce intermolecular crosslinking of the protein in solution during renaturation, the concentration of protein was generally maintained low. In order to obtain maximum yield of properly folded protein, the concentration of protein was kept at 1 ug/ml in the presence of 1 M guanidine.HCl after
10 sulphitolysis.

During renaturation of sulphonated proteins, a low concentration of denaturant was required. Maximal refolding of protein as indicated by the highest biological activity observed when the
15 guanidine.HCl concentration was in the range of 0.6 M to 2 M, above or below which the yield of properly refolded protein dropped precipitously.

The total concentration of glutathione and the ratio of the reduced and oxidized forms influenced
20 dramatically the rate of protein renaturation. The higher the glutathione concentration, the faster was the rate of refolding. The time to reach maximal refolding was reduced from 7 to 1 or 2 hours when the total glutathione concentration was increased
25 from 1.1 mM to 11 mM. However, in order to recover maximum yield of properly folded protein, the concentration of glutathione was kept at 1.1 mM. In refolding sulphonated proteins, maximal refolding was achieved when the ratio of the reduced to the
30 oxidized form of glutathione is maintained at ten.

During sulphitolysis, the effect of sodium sulphite and sodium tetrathionate concentrations on the rate of renaturation was analyzed. The optimal concentration of sodium sulphite and sodium tetrathionate was observed to be 20 mg/ml and 10 mg/ml respectively.

Temperature, in general, did not influence the rate of refolding of the catalytic fragments drastically. All the refolding experiments were carried out at room temperature. The optimal pH of the refolding after sulphitolysis was from 8.3 to 8.5.

The addition of non-ionic detergent to the refolding solution has been indicated to disperse the proteins so as to increase the yield of properly folded proteins. However, in all the experiments carried out, the data supported the fact that the addition of non-ionic detergent during folding is not necessary.

Purification of renatured catalytic fragment

Partial purification of F_B -CF and F_B - F_B -CF can be achieved by immunoaffinity chromatography (PAM-2). PAM-2 is a murine monoclonal antibody which will react with the two-chain m-TPA with a binding constant of M . After chromatography, a ten-fold increase in specific activity of F_B - F_B -CF can be achieved. Presumably, the column can selectively enrich biologically active molecules. On the other hand, no enhancement in specific activity of F_B -CF can be achieved by the PAM-2 chromatography.

Analysis of the partially purified F_B-F_B-CF and F_B-CF by SDS polyacrylamide gel electrophoresis indicated single bands of proteins at 41,000 and 31,000 daltons respectively. The protein is essentially pure because little or no contaminating bands are observed.

Characterization of recombinant tPA and analogues

The effect of plasminogen and fibrinogen on the activity of the catalytic fragments was determined (Table 1).

In the absence of plasminogen, no biological activity was detected. This indicates that the catalytic fragments are, indeed, plasminogen activators because they are plasminogen-dependent for activity.

Potentiation of the activity of natural tPA by fibrinogen has been clearly indicated. When compared to m-tPA, potentiation the activity of F_B-CF and F_B-F_B-CF by fibrinogen is less dramatic.

The biological activity of F_B-CF and F_B-F_B-CF can be completely neutralized by incubation with polyclonal antibody against m-tPA. This suggests that all the recombinant molecules are tPA analogues.

Table 1

Effect of Plasminogen and Fibrinogen on the
Activity of tPA and Catalytic Fragments

<u>Biological Activity (mu)</u>				
<u>Biological</u>	<u>complete</u> ¹	<u>-fibrinogen</u>	<u>-plasminogen</u>	<u>Potential₂ by fibrinogen</u>
5 FB-F _B -CF	12.5	6.0	0	2.7
FB-CF _B	67.5	35.0	0	1.8
m ^B tPA	96.0	7.0	0	8.2
urokinase	39.0	42.0	0	0

10 1 The plasminogen activator was incubated with both
 fibrinogen and plasminogen.

15 2 The potentiation of tPA activity by fibrinogen
 was estimated by the ratio of the tPA activity in
 the presence and absence of fibrinogen. Each
 number represents the average of a minimum of two
 separate experiments.

The molecular weights of tPA and the catalytic fragments were estimated by zymographic analysis. The molecular weights of r-tPA, CF, F_B -CF and F_B - F_B -CF were estimated to be 71,000, 27,500, 31,000 and 41,000 daltons respectively.

Binding to fibrin

Figure 8 indicates the extent of binding of F_B - F_B -CF to fibrin immobilized on microtiter plates. In contrast, much less F_B - F_B -CF binds to bovine serum albumin. In fact, BSA inhibits the non-specific binding of F_B - F_B -CF to the uncoated well of the 96-well plate. F_B - F_B -CF does not bind fibrinogen. In order to ascertain whether F_B - F_B is, indeed, the moiety responsible for the binding of F_B - F_B -CF to fibrin, a F_B tetramer was used to compete for binding. A molar ratio of 40 to 1 (Fb_4 to analogue) inhibited completely the ability of F_B - F_B -CF to bind to fibrin. On the other hand, melanoma-tPA, a very hydrophobic molecule, binds equally well to fibrin, BSA and the uncoated wells (Figure 9). Therefore, it is not possible to assess the binding capability of tPA to fibrin under these experimental conditions. These results also suggest the F_B - F_B -CF is less hydrophobic than the natural tPA molecules.

Half-life of F_B - F_B -CF

In order to determine the in vivo biological half-life of F_B - F_B -CF in rabbit, F_B - F_B -CF (0.8 mg per kilogram body weight) was injected into New Zealand White rabbits via the femoral vein. Via the femoral artery, blood samples were collected in 3.8% sodium citrate, pH 8.3, at 30 min intervals for three hours. After removing the blood cells by centrifugation, the F_B - F_B -CF activity in the plasma samples was determined by chromogenic assay, using S2251 as the substrate. The biological half-life of F_B - F_B -CF was estimated to be 93 minutes in rabbits. The biological half-life of natural tPA has been reported to be 10-12 minutes. (Beebe, D.P. and Aronson, D.L., Thrombosis Research 46, 663-674, 1984.)

Industrial Applicability

The tPA analogues of this invention are useful as thrombolytic agents. For this purpose, the tPA analogues can be formulated into injectable, thrombolytic compositions. A purified tPA analogue can be mixed with a conventional excipient for injection, e.g., a buffer, a filler, a stabilizer or the like. Preferably the analogues are administered intravenously; however, analogues comprising F_B domain of protein A linked to a catalytic fragment may be suitable for intramuscular administration because they are less hydrophobic than the natural molecule.

Equivalents

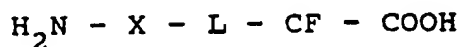
Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific

considered to be within the scope of this invention
and are covered by the following claims.

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CLAIMS

1. An analogue of tissue plasminogen activator (tPA) of the formula:



wherein CF is a catalytic fragment of a serine protease;

X, taken together with L, is a bond or X is a polypeptide which binds fibrin; and L is a bond or L is an oligopeptide linker joining X to CF.

2. A tPA analogue of Claim 1, wherein CF is the catalytic fragment spanning amino acid residues 262-527 of tPA and L and X taken together is a bond.

3. A tPA analogue of Claim 1, wherein X is the B domain of protein A.

4. An analogue of tissue plasminogen activator (tPA) of the formula:



wherein CF_{tPA} represents a catalytic fragment of tPA;

X represents a polypeptide of lower molecular

-50-

weight than the combined finger/kringle region of natural tPA having fibrin binding specificity; and

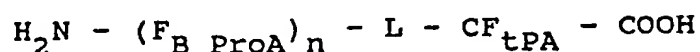
5 L represents a peptide bond linkage between X and CF_{tPA} or an oligopeptide linking X and CF_{tPA}.

- 10 5. A tPA analogue of Claim 4, wherein CF_{tPA} is the catalytic fragment spanning amino acid residues 262-527 of tPA or a fragment having substantially equivalent catalytic activity.
6. An analogue of Claim 4 wherein X is a prokaryotic leader segment.
- 15 7. A tPA analogue of Claim 4, wherein X is a fibrin binding domain of protein A present in single or multiple units.
8. A tPA analogue of Claim 7, wherein X is the B domain of protein A.
9. An analogue of Claim 3 wherein X is a eukaryotic leader segment.
- 20 10. A tPA analogue of Claim 3, wherein X is a fibrin binding domain of tPA.
11. A tPA analogue of Claim 4, wherein X is an antigen binding fragment of an immunoglobulin which specifically binds fibrin.

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12. A tPA analogue of Claim 4, wherein L is polypeptide susceptible to proteolytic cleavage.

5 13. An analogue of tissue plasminogen activator (tPA) of the formula:



wherein CF_{tPA} represents a catalytic fragment of tPA;

10 $\text{F}_\text{B ProA}$ represents the B domain of protein A or substantial equivalent thereof;

L represents a peptide bond or an oligopeptide linker separating F_B and CF_{tPA} ; and n is an integer from 1 to 5.

14. A protein analogue of Claim 13, wherein CF_{tPA} is the catalytic fragment spanning amino acid residues 262-527 of tPA.

15 15. A tPA analogue of Claim 13 having the formula:



16. A tPA analogue of Claim 13, having the formula:



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17. An analogue of tissue plasminogen activator (tPA) having thrombolytic and fibrin binding activities comprising:
 - 5 a. a catalytic fragment of human tPA linked as a contiguous polypeptide chain to:
 - b. a fibrin binding segment.
18. An analogue of claim 17 wherein the fibrin binding segment is prokaryotic.
- 10 19. An analogue of Claim 18 wherein the prokaryotic segment is the B domain of Protein A or multiple units thereof.
20. An analogue of claim 17 wherein the fibrin binding segment is eukaryotic.
- 15 21. An analogue of Claim 20, wherein eukaryotic is a fibrin binding region of an antifibrin antibody.
22. An analogue of tissue plasminogen activator (tPA), comprising:
 - 20 a. a catalytic fragment of human tPA linked as a contiguous polypeptide chain to:
 - b. a leader segment, the analogue having fibrinolytic and fibrin binding activities and having the following characteristics in comparison to natural tPA:
 - 25 i) increased solubility in an aqueous medium;

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- ii) more efficient expression in a host;
and
- iii) longer plasma half-life.

- 5 23. An analogue of tissue plasminogen activator of Claim 22, wherein the leader is prokaryotic in origin.
24. An analogue of tissue plasminogen activator of Claim 22, wherein the leader is eukaryotic in origin.
- 10 25. An analogue of tPA consisting essentially of a catalytic fragment of tPA linked to a prokaryotic fibrin binding segment.
26. A tPA analogue of Claim 25, wherein the prokaryotic binding fragment is the B domain of Protein A or multiple unit thereof.
- 15
27. DNA encoding a tPA analogue comprising a DNA sequence encoding a catalytic fragment of tPA optionally linked at the 5' end to a DNA sequence encoding a polypeptide having fibrin binding activity.
- 20
28. DNA of Claim 27, wherein the catalytic fragment is the portion of tPA encompassing amino acids 262-527 or a fragment having substantially equivalent catalytic activity.

29. DNA encoding a tPA analogue comprising, in 5' to 3' orientation:
- a) a first DNA sequence encoding an tPA polypeptide fragment having fibrin binding specificity or an exogeneous polypeptide having fibrin binding specificity linked to;
 - b) a second DNA sequence encoding a catalytic fragment of tPA.
30. DNA construct of Claim 29, wherein the second DNA sequence encodes the catalytic fragment of tPA spanning amino acid residues 262-527.
31. DNA construct of Claim 29, wherein the first DNA sequence encodes the heavy chain or light chain variable region of an immunoglobulin specific for fibrin.
32. A DNA construct of Claim 29, wherein the first DNA sequence encodes a single B domain of protein A or multiple sequentially aligned units of B domain of protein A.
33. DNA expression vector for expression of tissue plasminogen activator (tPA) analogues in bacterial cells, comprising in order:
- a promoter of bacterial or phage origin; a ribosome binding site; a translational start signal; a DNA fragment comprising a first DNA sequence encoding an tPA polypeptide fragment having fibrin binding specificity or an

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exogenous polypeptide having fibrin binding specificity linked to a second DNA sequence encoding a catalytic fragment of tPA; and a translational termination signal.

- 5 34. A DNA expression vector of Claims 33, wherein the promoter is the trp or tac promoter.
35. A DNA expression vector of Claim 34, wherein the first DNA sequence encodes the B domain of protein A.
- 10 36. An E. coli cell transformed by the vector of Claim 35.
37. A DNA expression vector comprising:
a trp or tac promoter, a ribosomal binding site, a translational start signal, a DNA
15 segment comprising a DNA sequence encoding a protein of the formula:
- $$\text{H}_2\text{N} - (\text{F}_\text{B ProA})_n - \text{CF}_{\text{tPA}} - \text{COOH}$$
- wherein $\text{F}_\text{B ProA}$ is the B domain of protein A; CF_{tPA} is a catalytic fragment of tPA and n is
20 1-5.
38. An E. coli transformed by the vector of Claim 39.

39. A synthetic gene encoding human tissue plas-
minogen activator (tPA) comprising codons
compatible with the E. coli codon bias and
containing two different, gene-unique recog-
5 nition sites for a restriction endonuclease
located so as to provide for endonuclease
cleavage of the gene into three fragments of
about equal length.
40. A synthetic gene encoding human tissue plas-
10 minogen activator having the deoxynucleotide
sequence shown in Figure 2, or substantial
equivalent thereof.
41. A method of isolating and renaturing hetero-
logous protein from inclusion bodies within
15 prokaryotic cells, comprising the steps of:
a. sedimenting the cells;
b. washing the cells in a buffer containing
20-40% sucrose;
c. washing the cells in a buffer containing
20 20-40% sucrose and a nonionic detergent;
d. lysing the cells in a buffer containing a
protease inhibitor;
e. sedimenting insoluble cellular components
of the lysed cells;
25 f. solubilizing the heterologous protein in a
buffer containing a strong denaturant;
g. sulphitolysing the solubilized protein in a
buffer containing the denaturant; and

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h. allowing the protein to renature for about 7-24 hours in a renaturation buffer containing the denaturant and reduced and oxidized glutathione in a molar ratio of about 10 to 1.

- 5 42. A method of Claim 41, wherein the cells are sedimented by centrifugation.
43. A method of Claim 41, wherein the detergent is sodium deoxycholate.
- 10 44. A method of Claim 41, wherein the strong denaturant is urea (2-8 molar) or guanidine-HCl (6-8 molar).

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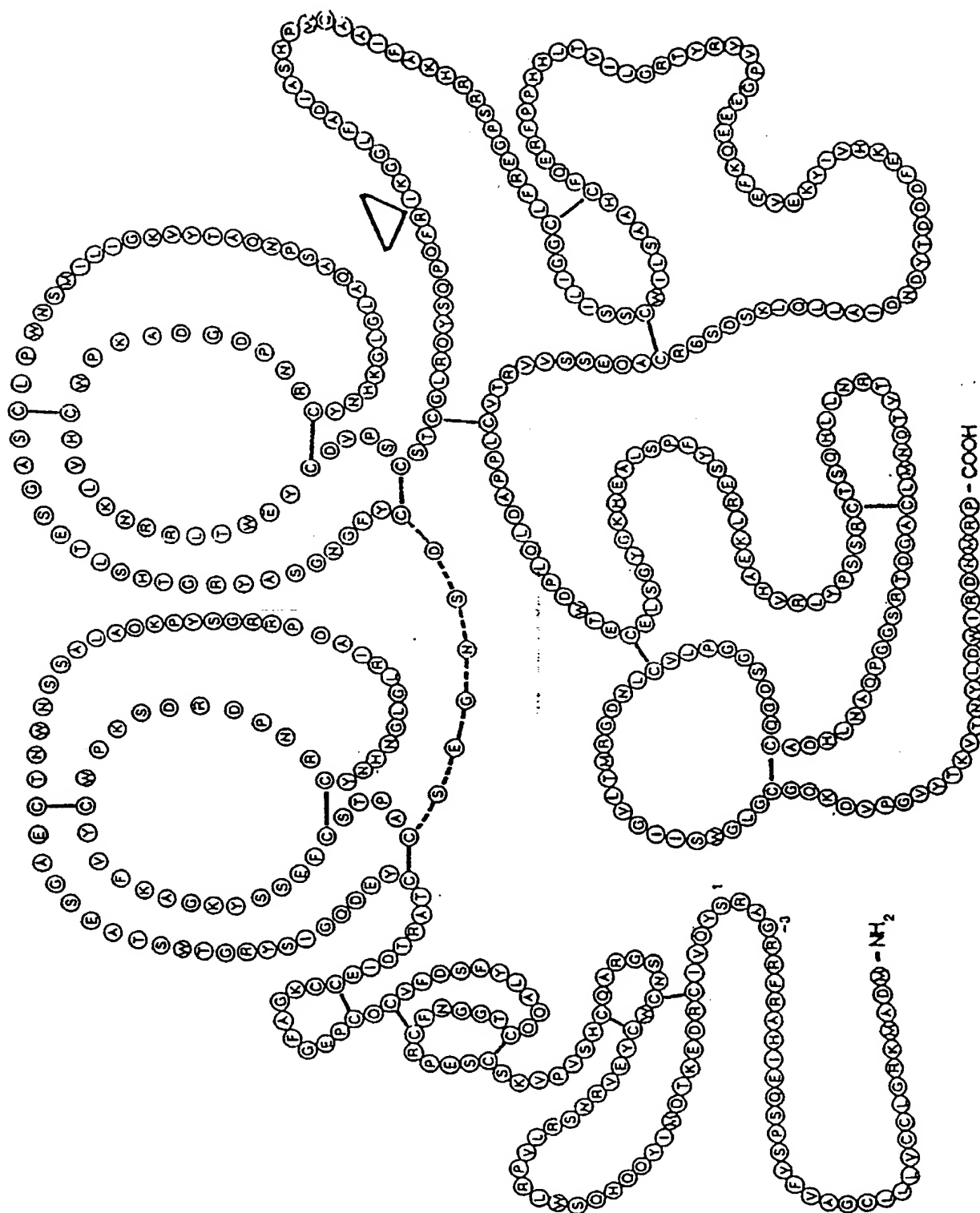


FIG. 1

Schematic two-dimensional model of the potential precursor t-PA protein including signal peptide and pro-sequence

SUBSTITUTE SHEET

Synthetic tPA Gene

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[illegible]

FIG. 2a

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 3/10
 CGATGACACTTACGACACGATATCGCTCTGCTGACAGCTGAAATCTGACTCTTCCCGTTGCGCTCAGGAGAGCTCCGTTGTACGCACTGTTTGGCTGCCG
 pAspAspThrTyrAspAsnAspIleAlaLeuLeuGlnLeuLysSerAspSerSerArgCysAlaGlnGluSerSerValValArgThrValCysLeuPro
 EcoRV PstI SacI N
 PvuII

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
 CCGGCCGACCTCCAGCTGCGGACTGGACTGAAATGCGAGCTGTCGCGCTACGGCAAGCACGAAGCTTTGTCTCCGTTCTACTCTGAACGCTTGAAGGAAAG
 ProAlaAspLeuGlnLeuProAspTrpThrGluCysGluLeuSerGlyTyrGlyLysIleGluAlaLeuSerProPheTyrSerGluArgLeuLysGluA
 ael PvuII BsaI HindIII
 EagI
 XbaII

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
 CTCACGTTCTCTGTATCCCGTCCCTCCCGTTGCACITCCCGACCTGCTGAACCTGACTGTAAACGACAACATGCTGTGCGCTGGTGAACACTCGTCTCTGG
 laHisValArgLeuTyrProSerSerArgCysThrSerGlnHisLeuLeuAsnLeuThrValThrAspAsnMetLeuCysAlaGlyAspThrArgSerGln

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
 CCGTCCGCGAGGCAACCTGCACGACGCAATGCCAGGGCGACTCTGGTGGCGCCGCTGGTTTGGCTGAACGACGGTCCGATGACTCTGGTTGGCATCATCTCT
 yGlyProGlnAlaAsnLeuIleAspAlaCysGlnGlyAspSerGlyGlyProLeuValCysLeuAsnAspGlyArgMetThrLeuValGlyIleIleSer
 RsrII SphI

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
 TGGGCGCTGGGCTGCGGTCAGAAAGACGTCCTCGGGCGTGTACACTAAGGTTACCACTACCTGGACTGGATTCTGTGACAACATGCGTCCGTAGGATCC
 TrpGlyLeuGlyCysGlyGlnLysAspValProGlyValTyrThrLysValThrAsnTyrLeuAspTrpIleArgAspAsnMetArgProAsn
 AatII SmaI BstXI BstEII
 XbaI BsaHI

FIG. 2b

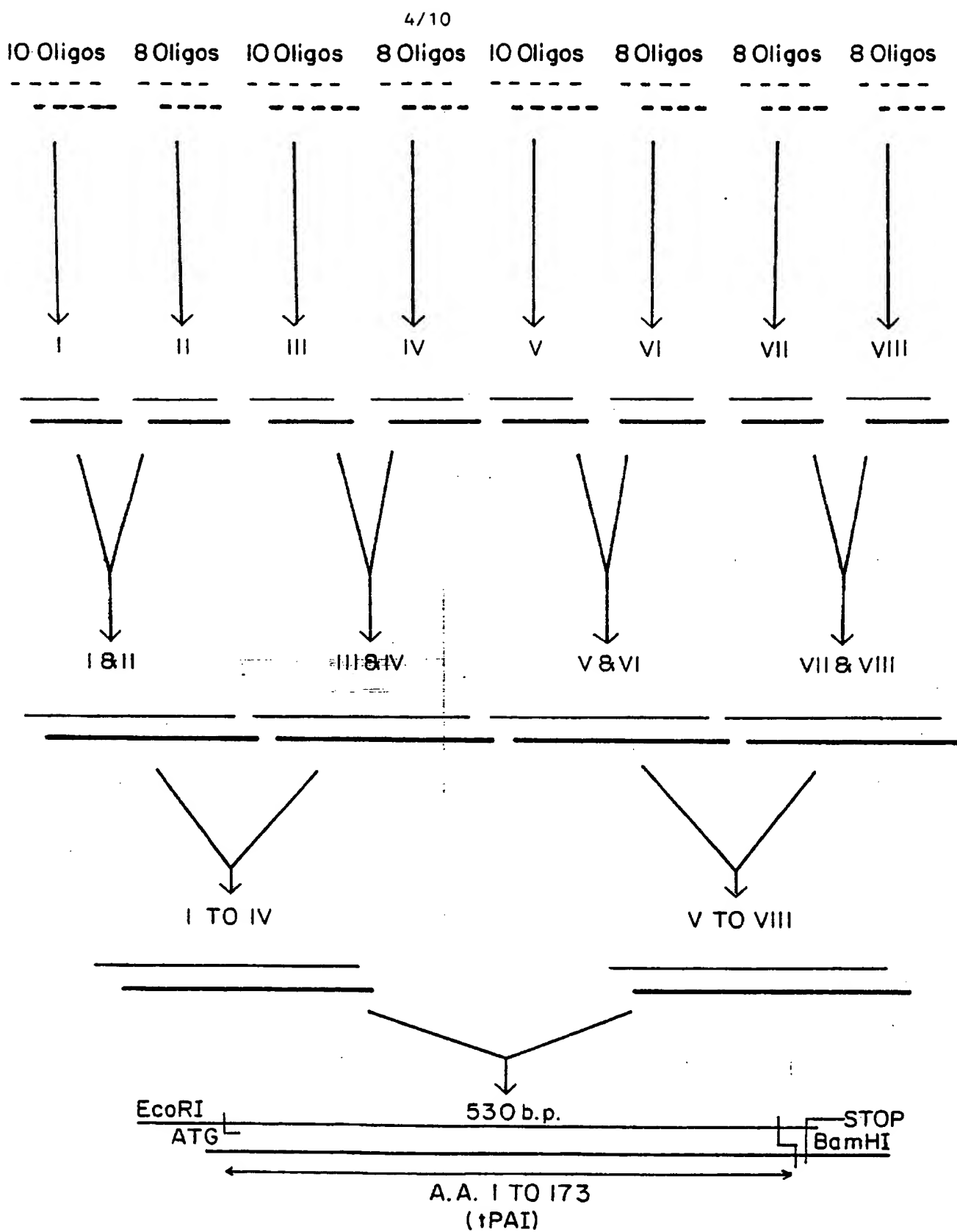


FIG. 3

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CONSTRUCTION OF EXPRESSION VECTORS IN E. COLI

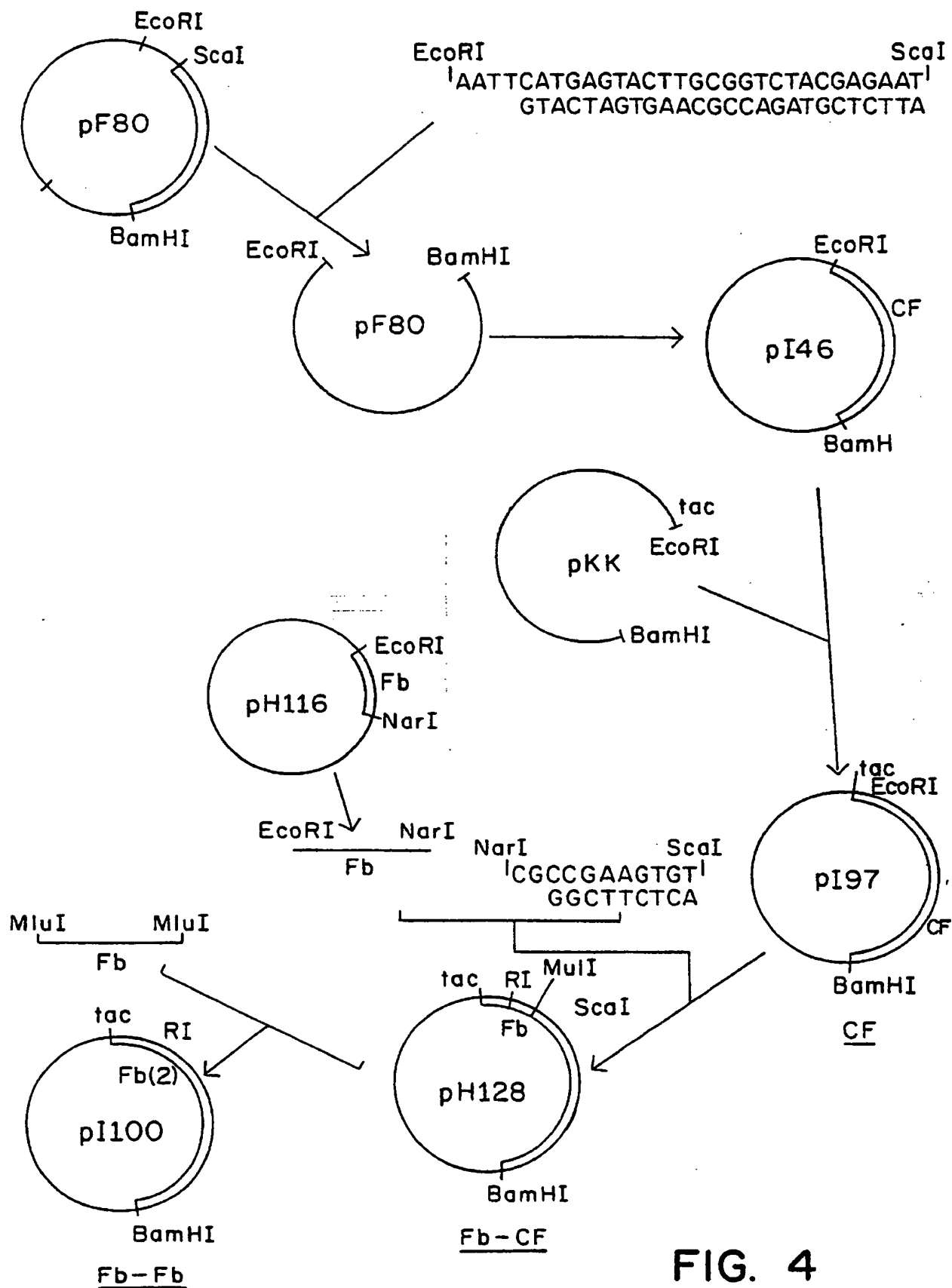


FIG. 4

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CONSTRUCTION OF t-PA ANALOGUE CONTAINING THE HYBRID KRINGLE

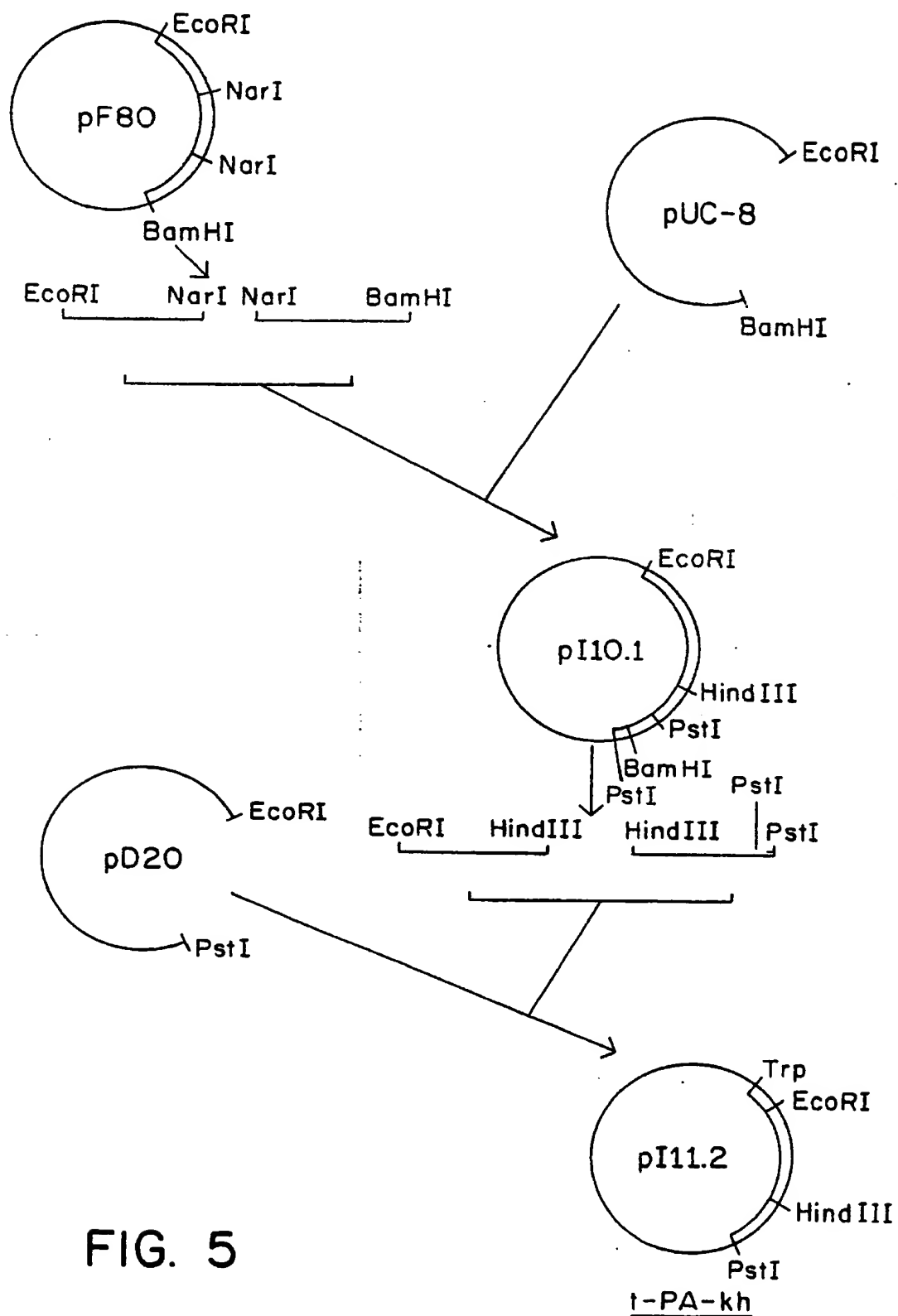


FIG. 5

CONSTRUCTION OF t-PA ANALOGUE CONTAINING THE FIRST KRINGLE 7/10

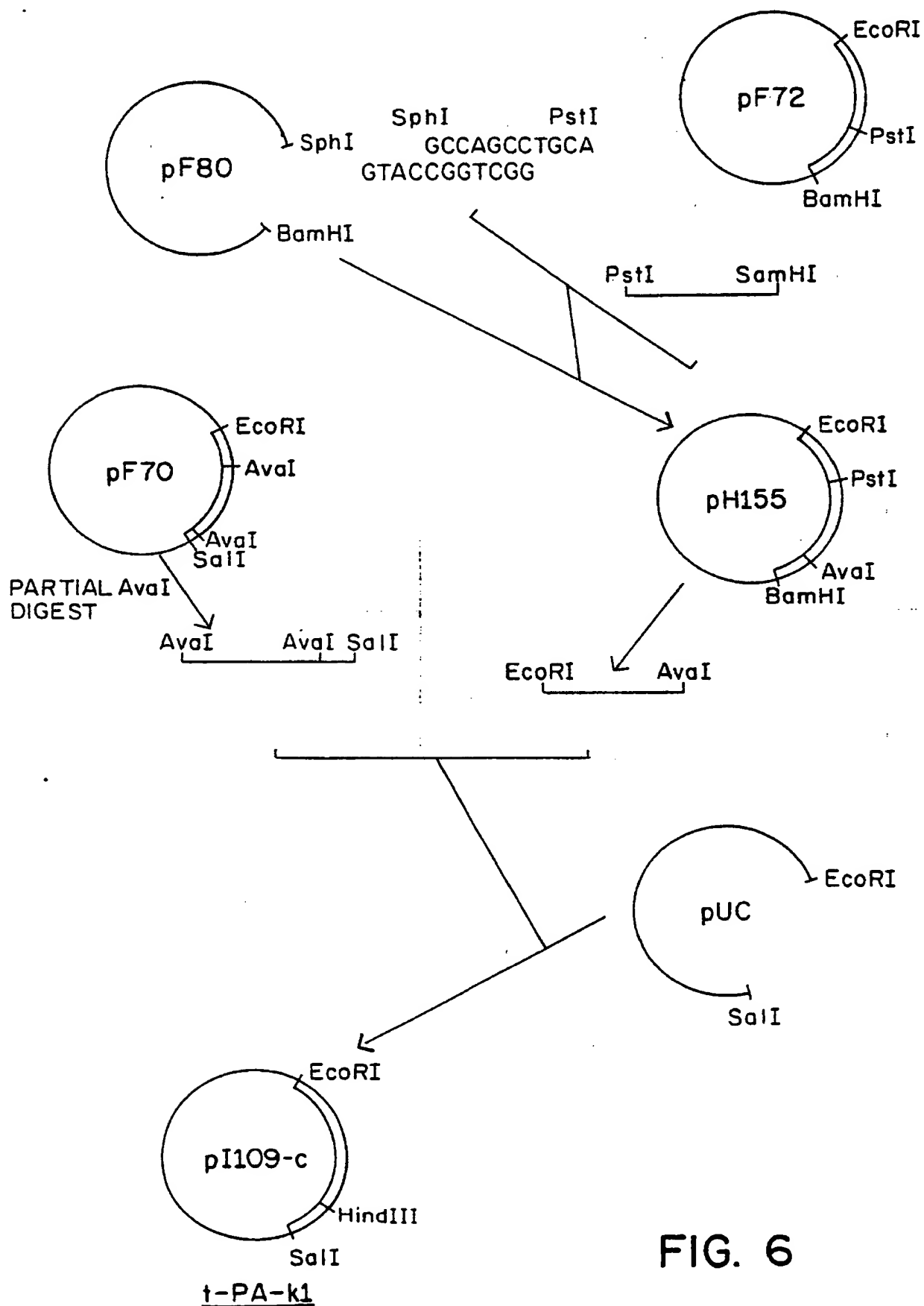


FIG. 6

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EcoRI HluI BglII
XbaI

HindIII		NarI	ScaI
---------	--	------	------

EcoRV

Bcll

Eaql	Snal	Asull
Xna111	Xna1	

EcoRV PstI : SacI EagI
 PvuII : : XbaI
 : : : XbaI

PvuII BsaI HindIII

Rsr11

Sphl

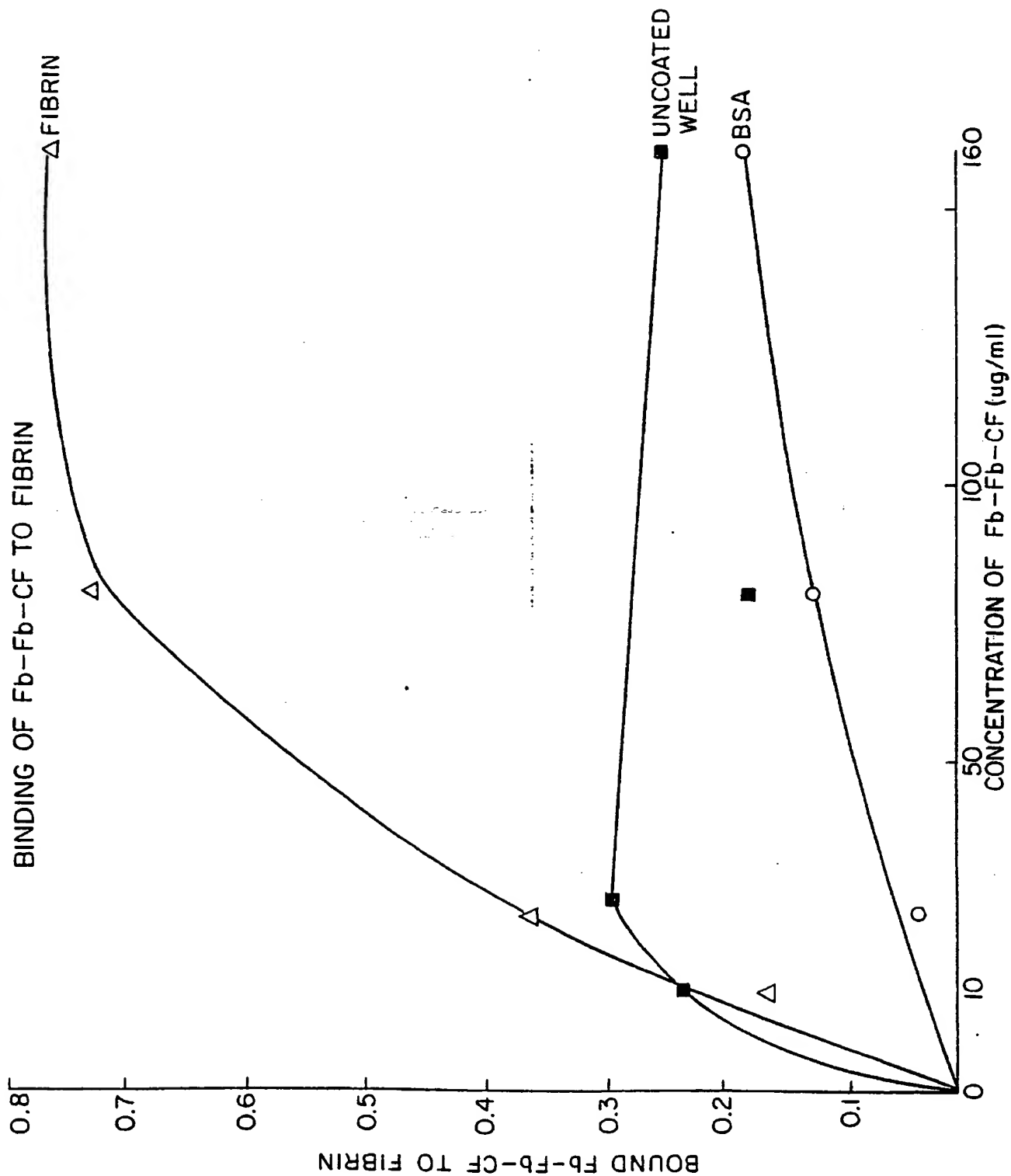
GlyCysGlyGlnLysAspValProGlyValIyrIhrLysValIhrAsnIyrLeuAspIrpIleArgAspAsnMetArgProIaaAsp
 AatIISmaI BstXI BamHI
 XmaI BstEII

FIG. 7

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FIG. 8



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BINDING OF mTPA TO FIBRIN

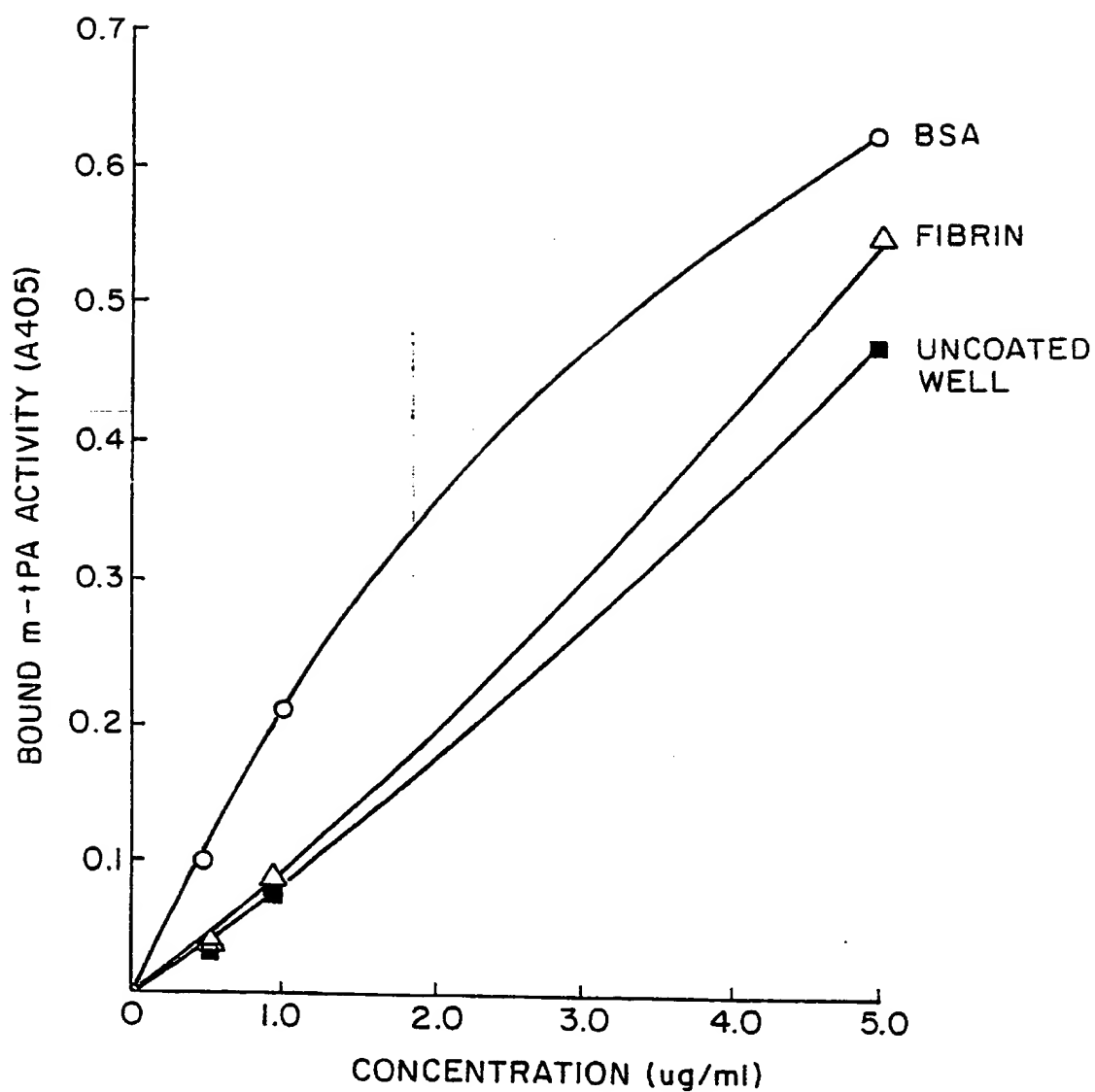


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 87/00760

I. CLASSIFICATION N F SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00; C 12 N 9/64; C 07 K 3/02; C 07 K 3/08														
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC⁴</td> <td style="padding: 5px;">C 12 N A 61 K</td> </tr> </table> <div style="border-top: 1px solid black; padding-top: 5px; margin-top: 5px;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸ </div>			Classification System	Classification Symbols	IPC ⁴	C 12 N A 61 K								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ¹⁰</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;"> Proceedings of the National Academy of Sciences of the USA, volume 83, no. 13, July 1986, (Washington, US), A.-J. Van Zonneveld et al.: "Autonomous functions of structural domains on human tissue-type plasminogen activator", pages 4670-4674 see introduction; figure 1; discussion <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1, 2, 4, 10, 17, 27, 29</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;"> EP, A, 0093619 (GENENTECH, INC.) 9 November 1983 see page 6, lines 25-35; page 9, lines 19-33 <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;"> FEBS Letters, volume 189, no. 1, September 1985, Elsevier Science Publishers B.V., (Biomedical Division), (Amsterdam, NL), H. Kagitani et al.: "Expression in E. coli of finger-domain lacking tissue-type plasminogen activator with high fibrin affinity", pages 145-149 see introduction; discussion; figure 2 <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Proceedings of the National Academy of Sciences of the USA, volume 83, no. 13, July 1986, (Washington, US), A.-J. Van Zonneveld et al.: "Autonomous functions of structural domains on human tissue-type plasminogen activator", pages 4670-4674 see introduction; figure 1; discussion <div style="text-align: center;">--</div>	1, 2, 4, 10, 17, 27, 29	Y	EP, A, 0093619 (GENENTECH, INC.) 9 November 1983 see page 6, lines 25-35; page 9, lines 19-33 <div style="text-align: center;">--</div>	1	Y	FEBS Letters, volume 189, no. 1, September 1985, Elsevier Science Publishers B.V., (Biomedical Division), (Amsterdam, NL), H. Kagitani et al.: "Expression in E. coli of finger-domain lacking tissue-type plasminogen activator with high fibrin affinity", pages 145-149 see introduction; discussion; figure 2 <div style="text-align: center;">--</div>	1
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center;">1st July 1987</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center;">28 AUG 1987</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">L. ROSSI </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center;">1st July 1987</div>	Date of Mailing of this International Search Report <div style="text-align: center;">28 AUG 1987</div>	International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">L. ROSSI </div>								
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International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">L. ROSSI </div>													

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A Proceedings of the National Academy of Sciences of the USA, volume 81, no. 17, September 1984, (Washington, US), T. Ny et al.: "The structure of the human tissue-type plasminogen activator gene: correlation of intron and exon structures to functional and structural domains", pages 5355-5359 see introduction; page 5358, column 1, line 18 - page 5359, column 1, line 28

1

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This international searching authority found multiple inventions in this international application as follows:

Claims 1-40: tPA analogues, DNA sequences coding for same and cells transformed by same.

Claims 41-44: Method of isolating and renaturing heterologous protein from inclusion bodies within prokaryotic cells.

See Form PCT/ISA/206 dated 10th June 1987.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-40

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international searching authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 87/00760 (SA 16842)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/08/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publicati date
EP-A- 0093619	09/11/83	GB-A, B 2119804	23/11/83
		FR-A, B 2526443	10/11/83
		DE-A- 3316297	17/11/83
		AU-A- 1414283	10/11/83
		JP-A- 59042321	08/03/84
		OA-A- 7419	30/11/84
		JP-A- 62091187	25/04/87
		AU-B- 563031	25/06/87

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82